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APPENDIX E: CHAPTER II.5.2 — NONTHROMBOGENIC TREATMENTS AND STRATEGIES

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INTRODUCTION

This chapter addresses the methods to improve the blood compatibility of biomaterials, the subject of a large body of literature in the biomaterials field and a topic of great importance clinically. To appreciate the contents of this chapter, a familiarity with material in Chapters I.2.12, I.2.17, II.2.6, II.3.5, and II.5.16D is expected.

HISTORICAL

In 1963, Dr. Vincent Gott at the Johns Hopkins University changed the field of biomaterials by failing to reproduce an earlier experiment. He was trying to show that an applied electric field could minimize thrombus formation on a metal surface. He obtained this result, but was somewhat mystified when he discovered that the wire leading to his negative graphite electrode was broken. He soon realized the importance of rinsing his electrode with a common disinfectant (benzalkonium chloride) and heparin prior to implantation. Thus, the first heparinized material was born. Robert Lehninger (Leininger et al., 1966) at Battelle Memorial Institute in Columbus, Ohio followed up with better quaternary ammonium compounds, and soon afterwards a host of chemical derivatization methods were devised to adapt the original GBH (graphite benzalkonium heparin) method to plastics and other materials. The principles underlying these strategies and others for lowering the thrombogenicity of materials are detailed here with examples.

CRITERIA FOR NONTHROMBOGENICITY

Thrombogenicity is defined (Williams, 1987) as the ability of a material to induce or promote the formation of thromboemboli. Here we are concerned with strategies to lower thrombogenicity, if not actually reduce it to zero, "nonthrombogenicity." Thrombogenicity should be thought of as a rate parameter, since low rates of thrombus or emboli formation are probably tolerable because the fibrinolytic or other clearance systems exist to remove "background" levels of thromboemboli. We are principally concerned with rates of thrombi formation that are sufficient to occlude flowpaths in medical devices (e.g., block the lumen of catheters) or rates of emboli formation that cause downstream problems such as myocardial infarction or transient ischemic attacks. The mechanisms of thrombogenicity are described in Chapter II.2.6, the methods to measure thrombogenicity are described in Chapter II.3.5, while the effects of fluid flow on thrombus development and embolization are described in Chapters II.3.5 and II.1.6.

Thrombi are produced through aggregation of activated platelets and/or the thrombin dependent polymerization of fibrinogen into fibrin. Thrombin is directly responsible for fibrin formation but it is also an important agonist of platelet activation. A simple model of thrombin generation is illustrated in Figure II.5.2.1a. The variety of mechanisms that lead to thrombin generation are lumped into a single parameter, k_p (cm/s), a rate constant that relates the rate of production of thrombin (per unit area), R_p (g/cm^{2}s), to the thrombin concentration at the surface of a material C_w (g/mL):

$$R_p = k_p C_w \tag{1}$$

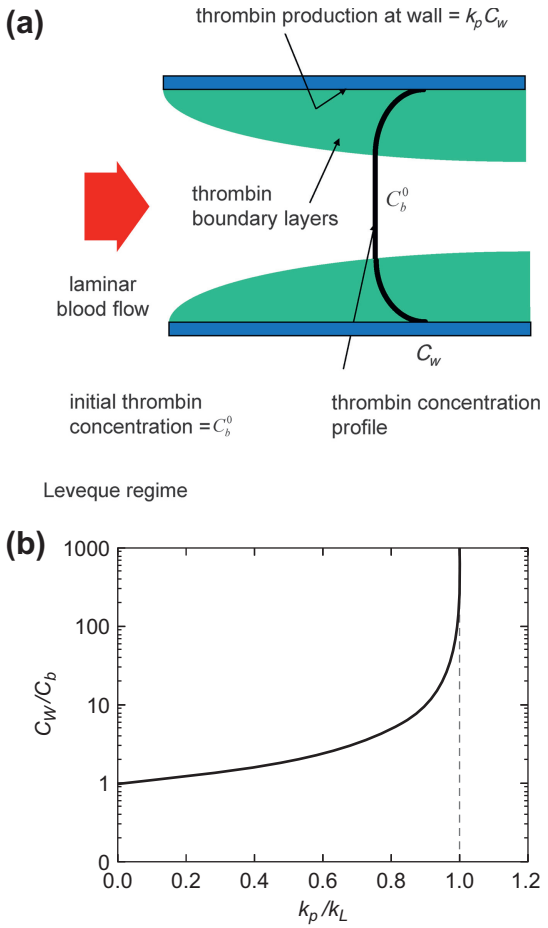


FIGURE II.5.2.1 (a) Model illustrating thrombin production at the surface of a tube as a balance between autoaccelerative production at the surface and mass transfer away from the wall. (b) Schematic illustration of Equation (2) showing the dramatic increase in wall to bulk concentration ratio as the mass transfer coefficient k_L becomes equal to the first-order autocatalytic production constant (k_p). Since k_L decreases with increasing axial position down a tube, increasing k_p/k_L corresponds to increasing x. *(Adapted from Rollason and Sefton, 1992.)*

k_p includes both the procoagulant effect of the material (via clotting factors and platelets) less any coagulation inhibition processes. A material balance (Basmadjian, 1990; Rollason and Sefton, 1992) equating the rate of production at the surface to the rate of transport away from the surface $k_L(C_w - C_b)$ for tubes greater than about 0.1 mm in diameter (Leveque region) gives:

$$\frac{C_w}{C_b^0} = \frac{1}{1 - k_p / k_L(x)} \qquad (2)$$

where C_b^0 is the concentration of thrombin at the inlet to a tube. $k_L(x)$ is the local mass transfer coefficient which is infinite at the tube inlet and decreases as one proceeds down the tube. Hence, C_w/C_b^0 increases progressively down the tube (Figure II.5.2.1b). When $k_L = k_p$, C_w becomes infinite, and a thrombus is expected. For a simple tube in laminar flow, k_L is in the order of 10^{-3} cm/s, and so k_p must be less than this to avoid a thrombus. Experimental results suggest that k_p is in the order of 10^{-3} for simple materials like polyethylene, but $<10^{-4}$ for heparinized materials (Rollason and Sefton, 1992). According to this model, only such low k_p materials can be expected to minimize thrombin production. This is one of the reasons heparinization and other active methods of inhibiting thrombin formation are so popular as strategies for imparting low thrombogenicity. There are, however, other criteria that must also be met.

It is also a requirement that platelet interactions with the surface do not lead to thrombosis. To some extent, platelet adhesion is inevitable. Once adherent, platelets change shape and release their granule contents which can activate bulk platelets. While it is intuitive to suggest that a nonthrombogenic surface should not support platelet adhesion it has not, unfortunately, been that simple. While most studies focus on evaluating the platelet compatibility of surfaces by measuring *in vitro* platelet adhesion, some *ex vivo* studies (Hanson et al., 1980; Cholakis et al., 1989) have demonstrated that even in the absence of adhesion, platelets can be "consumed." That is, the platelets are activated by the material leading to their premature removal from the circulation (Chapter II.3.5). This becomes apparent as a significant shortening of platelet lifespan, if not also a decrease in systemic platelet count. Whether this process is initiated by nonadhesive direct contact with the material or is the result of adherent platelet release, or even the effect of complement activation is currently unknown. Nevertheless, such an observation suggests that low platelet adhesion is not a sufficient criterion of *in vivo* platelet compatibility. Rather, low thrombogenicity is characterized by both low platelet adhesion and low platelet activation (see Chapter II.2.6 for a definition of activation); the latter may even be more important than the former. Leukocyte activation (expression of procoagulant activities such as tissue factor or CD11b upregulation; Gorbet and Sefton, 2004) and complement activation may also be key components in thrombogenicity (see Chapters II.2.4 and II.2.6), and in the future these too may become critical parameters defining the thrombogenicity of the surface. In an attempt to evaluate more than just platelet adhesion on biomaterials, flow cytometric techniques have been developed to measure bulk (or circulating) activated platelets, platelet microparticles, platelet–leukocyte aggregates and activated leukocytes (such as C11b upregulation) (Gemmell et al., 1995; Baker et al., 1998; Snyder et al., 2007). It is expected that such assays will result in better correlation between *in vitro* and *ex vivo*/*in vivo* performances.

While blood cells play an important role in material-induced thrombosis, concerns over in-stent thrombosis have renewed the interest in developing biomaterials to support and promote endothelialization. The potential thrombogenicity of these cells on a biomaterial add another dimension to this topic (McGuigan and Sefton, 2007).

INERT MATERIALS

Much of the effort in biomaterials research over the past 35 years has been directed towards the development of

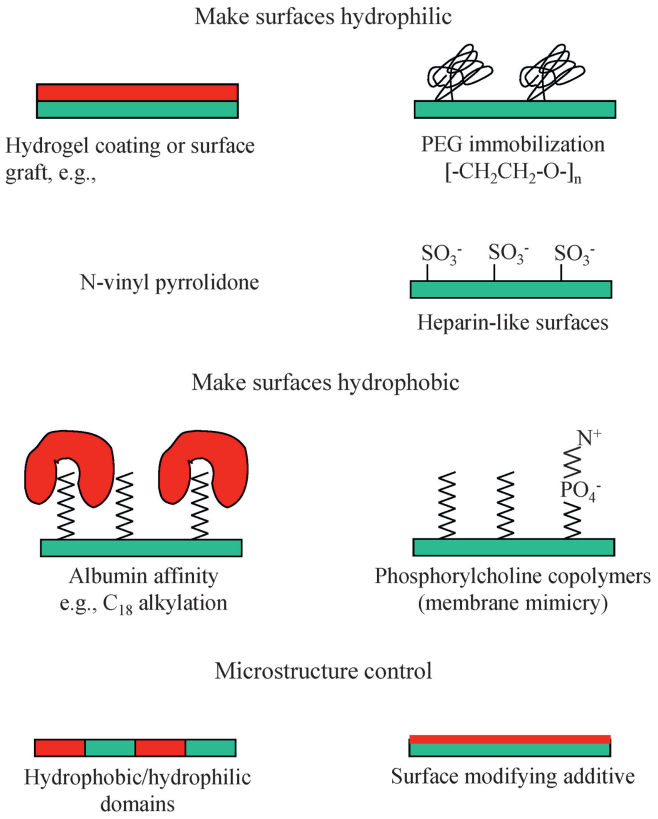


FIGURE II.5.2.2 Potential strategies for preparing inert surfaces with low thrombogenicity.

inert materials that do not react with platelets and coagulation factors. As outlined in Figure II.5.2.2, a number of approaches, often conflicting, have been developed. For example, there is still no consensus as to whether a surface should be hydrophilic or hydrophobic. The lack of agreement is largely due to our incomplete understanding of the biological pathways to materials failure and our inability to fully evaluate blood–material responses. In developing materials with lower thrombogenicity, researchers have primarily focused their efforts on modifying the surfaces of existing polymeric materials, like polyurethanes, silicone rubber, and polyethylene. This approach is reasonable, since it is only the surface chemistry of a material that should dictate its biological responses. As illustrated by the following discussion, the full range of surface modification strategies outlined in Chapter I.2.12 has been used, albeit with limited success. On the other hand, in comparing many plasma modified surfaces, Sefton et al. (2001) did not identify a modification chemistry that was superior to the base material in terms of platelet or leukocyte activation. Despite the successes in reducing protein and cellular deposits on some materials, a truly nonthrombogenic surface does not yet exist.

Hydrogels

A popular method to improve the blood compatibility of biomaterials is to increase surface hydrophilicity by incorporating a hydrogel at the surface. All commonly used hydrogels (see Chapter I.2.5) that can be cast, chemically cross-linked, or surface grafted have been used. By definition, hydrogels permit the retention of large amounts of water without dissolution of the polymer itself. This makes them similar to biological tissues, in that they are permeable to small molecules and possess low interfacial tension. It was Andrade who first postulated that since the interfacial free energy between blood and vascular endothelium was near zero, material surfaces which tend to have an interfacial energy of zero should have minimal thrombogenicity (Andrade et al., 1973). Today, considerable experimental evidence supports the claim that materials with minimal interfacial energy, like hydrogels, do not strongly support cell and/or thrombus adhesion. Unfortunately, such generalizations are always flawed by exceptions. A hydrogel (cellulose) used as a dialysis membrane material (Cellophane™) is well recognized as being thrombogenic, possibly because it is also a strong activator of the complement system (Chapter II.2.4). Furthermore, low adhesion (or low protein adsorption) is not the same as low thrombogenicity.

In the early 1980s, a number of polymers were grafted with hydrogel surfaces in an effort to decrease their thrombogenicity. For example, in a large *ex vivo* study (Hanson et al., 1980), a variety of surface grafted copolymers were prepared and evaluated for platelet consumption in a baboon shunt model of arterial thrombogenesis. Although few platelets were found adherent to the graft surface, the higher the water content, the greater the rate at which the graft tubing caused the destruction of the circulating platelets (Figure II.5.2.3). Even though this study concluded that hydrogels did not possess low thrombogenicity, utilization of hydrogels remains a popular approach to lower thrombogenicity. It is likely that platelet consumption will be of concern only in applications with large surface areas. On the other hand, platelet consumption is evidence of platelet

BOX 1 Associated Facts: Characterizing Thrombogenicity

Many investigators exploring methods of improving thrombogenicity rely on protein adsorption (especially fibrinogen) and platelet adhesion as surrogate markers of thrombogenicity to demonstrate the success of their modification. Surrogate markers are typically easier, indirect measurements of the potential for thrombus formation. However, these represent a limited assessment of the thrombogenicity of a surface, and imply a causative mechanism of action that is often not warranted. For example, the lack of platelet adhesion does not always correlate with the absence of platelet activation. *In vitro* and *in vivo* studies by Hanson et al. (1980), as well as Gemmell et al. (1995, 1997), have demonstrated significant platelet activation in the fluid phase in the absence of platelet adhesion. Many factors contribute to material-induced thrombogenicity (Gorbet and Sefton, 2004), and a limited understanding of mechanism means that assessing thrombogenicity requires more than looking at surface adsorption and adhesion.

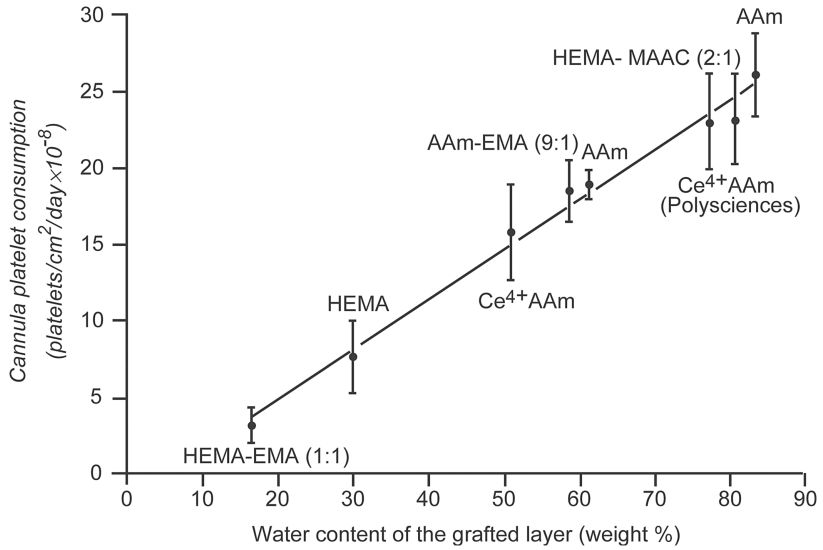


FIGURE II.5.2.3 Rate of cannula platelet consumption per unit area (in baboons) is directly related to the graft water content of shunts grafted with eight acrylic and methacrylic polymers and copolymers. HEMA: hydroxyethyl methacrylate; EMA: ethyl methacrylate; AAm: acrylamide; MAAC: methacrylic acid. Mean values 1 S.E. *(From Hanson et al., 1980, with permission.)*

activation, and the local (as opposed to systemic) consequences of such activation have yet to be defined.

Rather than radiation grafting of hydrogels onto materials, surfaces can also be simply coated with hydrophilic polymers, such as poly(vinyl pyrrolidone), PVP. While coating with PVP is intended to increase lubricity and ease catheter insertion, benefits on thrombogenicity (and bacterial adhesion) have been noted (Francois et al., 1996).

Polyethylene Glycol (PEG) Immobilization

Immobilization of the water-soluble synthetic polymer, polyethylene glycol (PEG, $-CH_2CH_2O-$), is a widely used approach to making a biomaterial surface more protein and cell resistant. It also makes the surface hydrogel-like. This approach was promoted by Edward Merrill, who recognized the lack of hydrogen bond donor sites on the PEG molecule and postulated that such sites might be involved with protein binding (Merrill and Salzman, 1983). A widely recognized theory that helps to explain the nonthrombogenicity of PEG-containing surfaces was presented by Nagaoka et al. (1984). They reasoned that the presence of diffuse hydrophilic polymers, covering a significant portion of a biomaterial surface, would exert a steric repulsion effect towards blood proteins and cells (Figure II.5.2.4). The dominance of steric repulsion over the van der Waals attractive forces was hypothesized to be dependent on the extension and flexibility of the polymer chain in the bulk solution. The resulting excluded volume effect results from a loss in configurational entropy of the PEG that in turn results from the rise in the local osmotic pressure occurring when PEG chains are compressed when blood elements approach the surface. This effect is dependent on both the chain length (N, monomers/chain), and the surface density

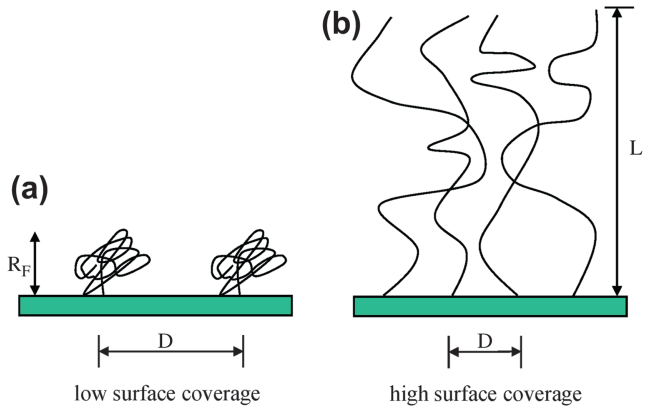


FIGURE II.5.2.4 Structure of polyethylene glycol (PEG) modified surfaces. (a) At low surface coverage ($D>>R_F$), molecules assume conformation and size similar to random coil. (b) At high surface coverage ($D<<R_F$), molecules are more extended, chain-like and form a dense brush. *(Adapted from de Gennes, 1980.)*

of chains (σ, number of chains per unit area). A simple scaling relationship relates these parameters (and a, the monomer size) to the thickness, L, of the polymer layer at the surface, for the case of a good solvent (specifically an athermal solvent), and when the chain density is high (distance between chains, D) is less than the Flory radius, R_F (de Gennes, 1980):

$$L \cong Na\sigma^{1/3} \tag{3}$$

Jeon and Andrade (1991), and among others, have theoretically modeled protein–surface interactions in the presence of PEG and concluded that steric repulsion by surface-bound PEG chains was largely responsible for the prevention of protein adsorption on PEG-rich surfaces. An extended discussion of PEG surface–protein interactions is presented in Chapter I.2.10, particularly

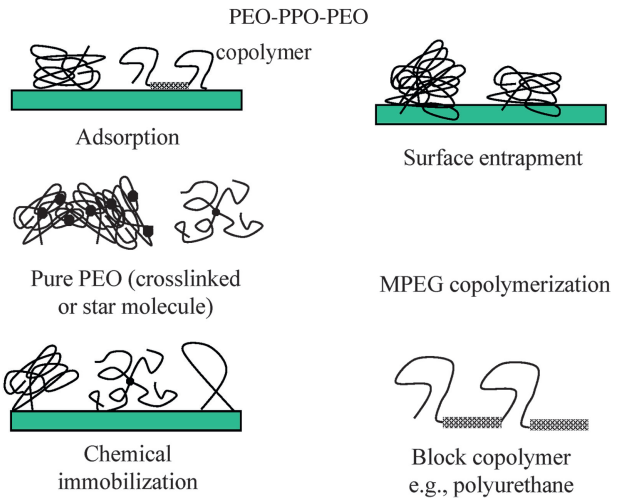


FIGURE II.5.2.5 Methods for incorporating polyethylene glycol (PEG) onto the surfaces of materials. PEO: poly(ethylene oxide); PPO: poly(propylene oxide); MPEG: methoxypolyethylene glycol methacrylate.

addressing the limited protein resistance of very short PEG chains where steric considerations are not relevant.

As shown in Figure II.5.2.5, a number of approaches have been used to enrich surfaces with PEG. For example, it has been grafted to surfaces via a backbone hydrogel polymer, such as in the preparation of methoxypolyethylene glycol monomethacrylate copolymers (Nagaoka et al., 1984). It has also been covalently bonded directly to substrates via derivatization of its hydroxyl end groups with an active coupling agent or, alternatively, the hydroxyl end groups have been reacted with active coupling agents introduced onto the surface (Desai and Hubbell, 1991a; Chaikof et al., 1992; Tseng and Park, 1992). The commercial availability of a large number of reactive PEG molecules (e.g., amino-PEG, tresyl-PEG, N-hydroxysuccinimidyl-PEG) has greatly facilitated the use of covalent immobilization strategies. Unfortunately, it is difficult to achieve the required high surface coverages by immobilization, since the first molecules immobilized sterically repel later molecules that are attached, unless thermodynamically poor solvents are used. The surface fraction may then be too low to completely "mask" the other functional groups that may be present. Pure monolayers of star PEG have been grafted to surfaces in an attempt to increase surface coverage (Sofia and Merrill, 1998), and radio frequency plasma polymerization of tetraglyme has been used to the same end (Shen et al., 2001). Other investigators have used block copolymers (e.g., Pluronic™) of PEG and PPO [poly(propylene oxide)] by adsorption, gamma irradiation, or as an additive (McPherson et al., 1997); some have combined PEG with other strategies such as phosphorylcholine (Kim et al., 2000), cyclodextrin (Zhao and Courtney, 2007) and negatively-charged side groups (from vinyl sulfonic acid sodium salt) (Lee et al., 2004).

PEG has also been incorporated, by both ends, into polyetherpolyurethanes (Merrill et al., 1982; Okkema et al., 1989). Unfortunately, the results in this case depend on a combined effect of surface microphase separation and the hydrogel (hydrated chain steric exclusion) effect of the PEG side chains. While some have noted lower thrombogenicity, others have not. For example, Okkema et al. (1989) synthesized a series of poly(ether-urethanes) based on PEG and poly(tetramethylene oxide) (PTMO) soft segments, and noted that the higher PEG-containing polymers were more thrombogenic in a canine *ex vivo* shunt model. Since PEG-containing polyurethanes have a considerable non-PEG phase, Chaikof et al. (1989) prepared a cross-linked network of PEG chains using only small polysiloxane units.

A number of investigators have noted that the beneficial effect of PEG is molecular weight dependent. Nagaoka et al. (1984) were one of the first to demonstrate that increasing the PEG chain length of hydrogels containing methoxy poly(ethylene glycol) monomethacrylates led to reductions in protein and platelet adhesion (Figure II.5.2.6). To some extent, however, the benefit of high molecular weight PEO[1] is compromized by the crystallizability of long chain PEO. In some cases, the benefit of longer chains may reflect particular process advantages (such as surface entrapment). Chaikof et al. (1992), with end-linked PEO, and Desai and Hubbell (1991b), with physically entrapped PEO, found lowest protein and platelet or cell deposition with high molecular weight PEO (>18,000 Da). However, when studying PEG-modified polyurethanes, Tan and Brash (2008) noted the importance of PEG surface density over length: the shortest PEG chain (MW: 550) allowed for a higher surface density and provided the highest reduction in protein reduction compared to modification with longer chains of PEG (MW 2,000 and 5,000).

It is clear that incorporation of PEG results in reduced levels of cell (including platelet) adhesion and protein adsorption when compared to unmodified and typically hydrophobic substrates. It is far less clear whether the reduced adhesion or adsorption translates to lower material thrombogenicity (Llanos and Sefton, 1993a,b). Further, it is not clear whether reduced adhesion/adsorption is due specifically to the thermodynamic effects of PEG or PEO, or to the increase in surface hydrophilicity after its immobilization. While the *in vitro* results have looked promising, the lack of correlation between the few *in vitro* and *ex vivo* studies is of concern. More recent efforts with plasma deposited tetraglyme (Shen et al., 2001) have led to surfaces with ultra-low adsorbed fibrinogen, suggesting that previous attempts at using PEG modification have not been successful because of the inability to achieve the desired ultra-low (<5 ng/cm^2) levels of adsorbed protein. On the other hand, more recent studies from the same group (Zhang, et al., 2008; Zhang and Horbett, 2009) show that such surfaces are

[1]PEG >10 kD is called PEO [poly(ethylene oxide)], reflecting the different monomer and polymerization process used.

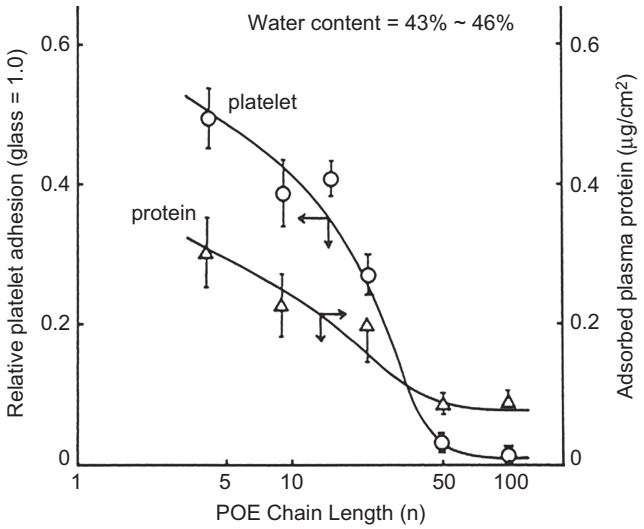


FIGURE II.5.2.6 Effect of PEG (or "POE") chain length (*n*) on the adhesion of platelets (from activated platelet rich plasma) and adsorption of plasma proteins (from plasma) onto poly(methyl methacrylate co-methoxypolyethylene glycol methacrylate) gels. Mean values and standard deviation are shown. Both protein adsorption and platelet adhesion were measured by total protein anaylsis. A relative platelet adhesion value of 0.3 corresponded to an Scanning Electron Microscopy (SEM) platelet adhesion density of ~80 platelets/1,000 μm². *(From Nagaoka et al., 1984, with permission.)*

not as resistant to protein adsorption as expected when exposed to physiological conditions (undiluted plasma). Low amounts of adsorbed von Willebrand Factor (vWF) (and not fibrinogen only) also played a significant role in platelet adhesion under high shear rates. There is also new evidence that properties of PEO and PEG surfaces may also be affected by the sterilization process (see Box 2). This highlights the importance of testing modified surfaces under physiological conditions before drawing conclusions from *in vitro* studies using (surrogate) markers such as platelet adhesion or fibrinogen adsorption.

Albumin Coating and Alkylation

The early observation that surfaces coated with albumin did not support protein adsorption and platelet adhesion (reviewed by Andrade and Hlady, 1986) led many investigators to lower material thrombogenicity by either albumin coating or enhancing the affinity of albumin for surfaces via alkylation. It is thought that albumin adsorption lowers thrombogenicity, since it does not possess the peptide sequences to enable interaction with cells (platelets and leukocytes) or the enzymes of the coagulation cascade. It adsorbs relatively tightly onto hydrophobic surfaces, while having a poor affinity for hydrophilic surfaces. Consequently, utilization of this strategy entails increasing the hydrophobicity of the surface, which is the opposite of the above discussed strategies whereby the hydrophilicity of the surface was increased. Albumin coating is consistent with another hypothesis, developed early in the 1970s, which suggested that hydrophobic surfaces with critical surface tensions (conceptually related to surface free energy) around 20–30 dynes/cm would have a lower thrombogenicity (Baier et al., 1970).

A limitation of relying on albumin coating is that other proteins will adsorb to the surface displacing the albumin, limiting the long-term effectiveness of this strategy (see Chapter II.1.2). To prevent displacement, glutaraldehyde cross-linking (Kottke-Marchant et al., 1989) or covalent immobilization has been used (Hoffman et al., 1972; Matsuda and Inoue, 1990). Reduced platelet adhesion was observed *in vitro* with a multilayer of albumin on polyethersulfone deposited using a layer-by-layer technique (Sperling et al., 2006). Since albumin has binding pockets for long alkyl chains, Munro et al. (1981) demonstrated that surfaces with long carbon chains (C-16 or C-18) have a high affinity for albumin, and provided for a dynamically renewable natural albumin layer. Albumin adsorption (from diluted plasma) was enhanced on alkylated cellulose membranes (Frautschi et al., 1995). Butylation (but not longer alkyl chains) of polyvinyl alcohol hydrogel also dramatically reduced platelet reactivity in a dog shunt (Strzinar and Sefton, 1992) suggesting that some of the observed benefit was due to the creation of a hydrophobic surface (Duncan et al., 1997).

BOX 2 Associated Facts: Effect of Sterilization Procedure on Hydrogel Properties

Most *in vitro* blood experiments are completed in two hours or less and little consideration is given to sterilization. However, sterilization may affect the materials' surface properties and thrombogenicity. For example, sterilizing PEG hydrogels using current methods (ethylene oxide, gamma, or plasma) may result in changes in surface roughness, protein adsorption, or the presence of radical species (Lleixa et al., 2008; Kanjickal et al., 2008). These changes lead to significant differences in the biocompatibility response between unsterilized and sterilized samples. Thus, it is important to test a surface-modified material in a "final product state," since processing may significantly alter its ability to reduce thrombogenicity *in vivo*. Removing endotoxin is another important consideration, at least in understanding the role of leukocytes and endothelial cells (Gorbet and Sefton, 2005a).

Zwitterionic Group/Phospholipid Mimicking Surfaces

A number of investigators have hypothesized that a surface similar to the external zwitterionic outer phospholipid membrane of cells should be nonthrombogenic.[2] Since phosphorylcholine (PC) is the major lipid head group

[2]Upon cell activation, the negatively-charged phospholipids that are preferentially located on the cytoplasmic face of the cell membrane flip to the outer membrane, which accelerates blood clotting by enabling assembly of the prothrombinase and tenase complexes.

on the external surface of blood cells and red blood cells are inert in coagulation assays, PC has been the choice of many investigators for incorporation into surfaces.

Durrani et al. (1986) prepared a series of reactive derivatives of phosphorylcholine that were designed to react with surface hydroxyl groups and surface acid chlorides on various materials. Another approach involved coating materials (Campbell et al., 1994; Lewis et al., 2000; Iwasaki et al., 2002), or blending a polyurethane (Ishihara et al., 1995, 1999) with a methacryloylphosphorylcholine (MPC; Figure II.5.2.7)/polyacrylate copolymer. Platelet adhesion was significantly reduced on phosphorylcholine coated expanded polytetrafluoroethylene (ePTFE) grafts at 90 minutes in dogs, and anastomotic neointimal hyperplasia and neointimal cell proliferation were also reduced (Chen et al., 1998). Ishihara et al. (1998) have attributed the low protein adsorption of their phospholipid polymers to the high free water fraction, a mechanism that has been confirmed by other investigations (Chen et al., 2005). A further modification has involved grafting only the polar part of phosphorylcholine onto ammonia plasma treated ePTFE to promote more of the red blood cell-like properties while preventing lipid uptake (Chevallier et al., 2005). This led to significant reduction in both platelet and leukocyte adhesion.

A recent approach has also been to modify materials (for example polyurethane) using zwitterionic polymers/monomers such as carboxybetaine or sulfobetaine (Figure II.5.2.8a,b) to mimic the cell membrane (Yuan et al., 2003; Jiang et al., 2004; Kitano et al., 2005). Zhang et al. (2008) prepared polymeric brushes containing zwitterionic groups by surface initiated atom transfer radical polymerization using sulfobetaine methacrylate (polySBMA) and carboxybetaine methacrylate (polyCBMA) on gold surfaces. Not only did such zwitterionic polymeric brushes exhibit superlow protein adsorption, with the polyCBMA brushes adsorbing less than 0.4 ng total protein/cm^2 (experiments performed in 100% plasma), but significantly reduced platelet adhesion was also observed. The polyCBMA also demonstrated unique anticoagulant properties, suggesting that such a material may offer excellent blood compatibility, although the mechanism by which polyCBMA is superior to polySBMA is currently unclear.

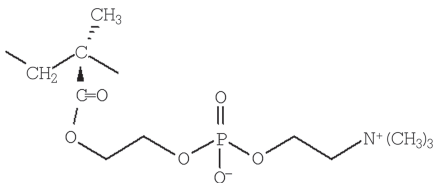


FIGURE II.5.2.7 Chemical structure of poly(2-methacryloyloxyethyl phosphorylcholine).

Surface Modifying Additives (SMAs)

The blending of a copolymer, composed of polar and nonpolar blocks, to a base polymer appears to be a successful means to lower material thrombogenicity. The strategy, originally developed by Thoratec Laboratories Inc., is a means to alter the surface properties of materials without affecting bulk properties (Ward et al., 1984). The copolymers, added in low concentration, migrate to the base polymer surface during and after fabrication, and dramatically change the outermost surface molecular layers that comprise the region that determines biocompatibility (Tsai et al., 1994). The copolymers have a structure which is amphipathic, that is, certain groups or segments will have an attraction for the continuous phase (major polymer component of the blend), while other portions of the molecule will have little attraction for the base polymer and will be of lower polarity (Ward et al., 1984) (see Chapter I.2.12). They have been used to lower the thrombogenicity of cardiopulmonary bypass and hemodialysis components by using SMA blended polymers or SMA coated surfaces. A clinical evaluation of the effects of SMA on cardiopulmonary bypass circuits demonstrated a reduction in platelet interactions with no effect on complement activation (Gu et al., 1998).

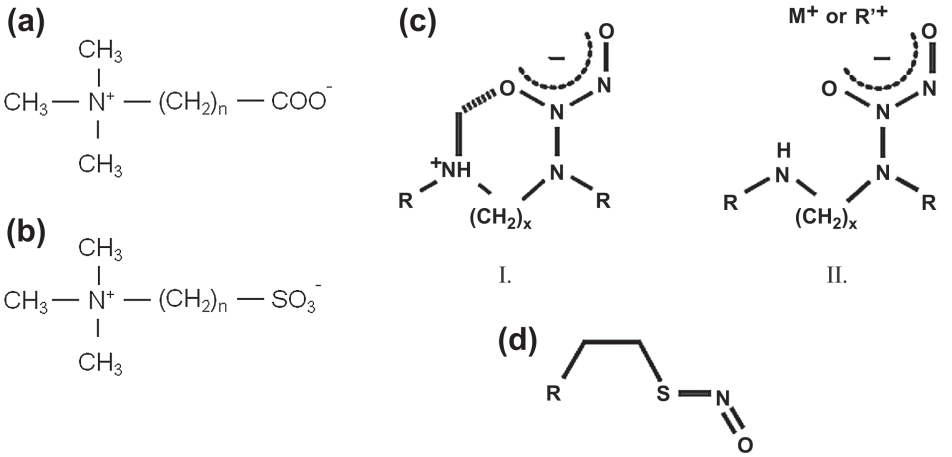


FIGURE II.5.2.8 Generic structures of zwitterionic molecules (a: carboxybetaine and b: sulfobetaine) and two common NO donors: (c) zwitterionic (I) and cation-stabilized (II) diazeniumdiolates, and (d) an S-nitrosothiol. *(From Frost et al., 2005.)*

Fluorination

The incorporation of fluorine into materials is also a strategy to lower thrombogenicity. Polymers with fluorinated chains are highly hydrophobic, and it is believed that the fluorine group's low surface energy modulates protein adsorption and platelet adhesion/activation. Preparing fluorine-rich surfaces is often facilitated by the tendency of fluorine-containing functional groups to concentrate on the surface of the polymer during preparation. Kiaei et al. (1988) found a strong effect of a fluoropolymer plasma on thrombus formation and plasma-induced graft polymerization of a fluorocarbon on polyethylene also reduced platelet adhesion (Lin et al., 2000). Fluoroalkyl groups as chain extenders have also been used to prepare nonthrombogenic polyurethanes (Kashiwagi et al., 1993). Using a similar approach, Wang and Wei (2005) showed that increasing fluorine content in polyurethanes by decreasing soft segment length resulted in a decrease in platelet adhesion and activation (of adherent platelets). Fluorinated surface-modifying macromolecules (SMMs) used to increase polyetherurethane biostability also improved blood compatibility (Jahangir et al., 2002), although the effect does not appear to be directly correlated with a decrease in fibrinogen adsorption (Massa et al., 2005).

Heparin-Like Materials

A number of synthetic polymers have been synthesized or modified in order to prepare polymers with chemical similarity to heparin, and thus possess heparin-like activities. For example, Fougnot et al., (1983) synthesized sulfonate/amino acid sulfamide polystyrene derivatives in order to create insoluble heparin-like materials. Some investigators (e.g., Grasel and Cooper, 1989) only incorporated sulfonate groups in an effort to lower thrombogenicity by surface thrombin inhibition. Enhanced binding and inactivation of thrombin was found, suggesting that these weak heparin-like molecules are sufficiently dense to lower thrombogenicity. A newer approach combined sulfonation and PEG-like materials in the use of sulfonated cyclodextrin polymers (Park et al., 2002); and improved blood compatibility was recently seen in a canine shunt model (Han et al., 2006).

Self-Assembled Surface Layers

Self-assembled surface layers (Whitesides et al., 1991) have been envisioned as useful templates to nucleate or organize ordered, designed biomaterials (Ratner, 1995; see Chapter I.2.12). Self-assembled monolayers (SAMs) of alkylsilanes supported on oxidized polydimethyl siloxane (PDMS) rubber have been used as a model system (Silver et al., 1999). The authors reported that surfaces grafted with hydrophobic head groups (CH_3 and CF_3) had significantly lower platelet and fibrinogen deposition than the surfaces composed of hydrophilic head groups in a canine *ex vivo* arteriovenous series shunt model. In a later *in vitro* study (Sperling et al., 2005), contradictory results were obtained whereby SAMs with $-CH_3$ exhibited the most platelet adhesion compared to –COOH and –OH. Reduced TAT formation was observed for $-CH_3$ and –COOH. These studies highlight the difficulty of generalizing the effect of surface modification on blood compatibility. In addition to producing zwitterionic polymeric brushes (see Section "Zwitterionic Group/Phospholipid Mimicking Surfaces"), Zhang et al. (2008) produced a series of SAM surfaces using oligoethyleneglycol, phosphorylcholine, oligophosphorlycholine, mixed $SO_3^-/N^+(CH_3)_3$ and mixed $COO^-/N^+(CH_3)_3$; there was no significant difference in platelet adhesion or fibrinogen adsorption among these SAMs.

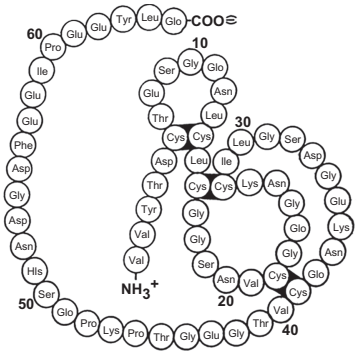
ACTIVE MATERIALS

The limited success of the various surface treatments for lowering thrombogenicity has encouraged researchers to pursue other strategies. The most popular strategy, and the one that started the field, is the heparinization of surfaces, although the incorporation of other antithrombotic and antiplatelet agents into materials is gaining popularity (Figure II.5.2.9). Currently, the antithrombotic agents utilized have mainly been against thrombin (hirudin, curcumin, thrombomodulin).

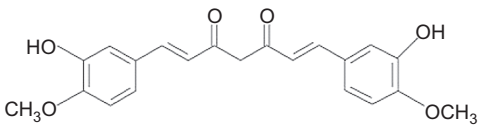
The incorporation of antiplatelet agents into materials has been limited by the instability and complex mechanism of action of these agents. Platelet GPIIb/IIIa antagonists (e.g., ReoPro, Centocor) and agents against factor Xa (low molecular weight heparins, LMWH) or inhibitors of tissue factor expression (extrinsic pathway) are currently being used systemically during cardiovascular surgery. There is potential for such drugs to be included in novel strategies to modify materials.

Heparinization

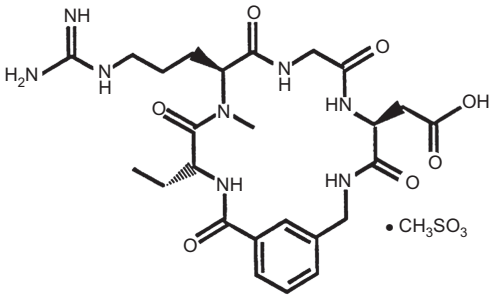
Heparinization of surfaces continues to be the most popular technique for lowering the thrombogenicity of materials. As illustrated in Figure II.5.2.10, heparin is a linear, acidic carbohydrate composed of repeating disaccharide units that are O- and N-sulfated. The molecular weights of heparin chains range from 1,200 to 40,000 Daltons, with a mean molecular weight of approximately 10,000. Depending on its molecular weight and structure, it can inhibit, in association with its cofactor (antithrombin III), the serine proteases: thrombin and factor X. Since thrombin is also a potent platelet activator, binding it into an inactive complex should help minimize platelet activation. Investigators have been able to covalently, ionically, and physically attach heparin to various substrates utilizing a number of chemistries. The main concern has been that once immobilized, the heparin should be able to assume its native conformation, and be able to interact with antithrombin III.



Hirudin



Curcumin



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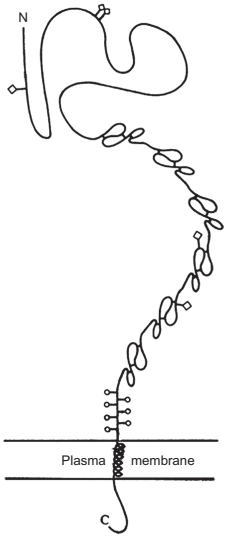
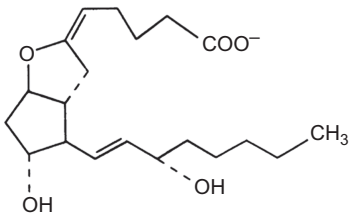


Thrombomodulin



Prostacyclin

FIGURE II.5.2.9 Agents, other than heparin, that have been incorporated into biomaterials to actively control thrombogenicity. Hirudin and curcumin are thrombin inhibitors. Prostacyclin (PGI2) is a natural inhibitor of platelet function while DMP728 is a small molecule GPIIb/IIIa antagonist. Thrombomodulin inhibits thrombin by activating protein C.

The effectiveness of heparin as an agent capable of increasing synthetic venous graft patency and reducing downstream anastomotic neointimal hyperplasia and cell proliferation was demonstrated using a novel polytetrafluoroethylene-based local drug delivery device. Heparin was infused adjacent to the graft wall and at downstream anastomotic sites for 14 days and demonstrated effectiveness (Chen et al., 1995).

Ionically Bound Heparin and Controlled Release Systems. If negatively-charged heparin is bound ionically to the surface, then heparin will be slowly released over time due to exchange with the blood. A similar effect is obtained if heparin is dispersed within a hydrophobic polymer. Selected techniques to produce materials which release heparin at biologically significant rates have been discussed in depth by a number of investigators (Sefton et al., 1987; Amiji and Park, 1993). Ionic approaches involve binding the highly negatively charged heparin onto a cationic surface through ionic binding. The limitation is that the leaching of heparin will eventually leave the surface unprotected. Certain non-ionic approaches are also characterized by high release rates (due to relatively unstable bonds), so that determining heparin release rate (in addition to amount bound) is a primary means of characterizing these surfaces. Limitations to the use of dispersed systems include the loading capacity of heparin, which prevents long-term usage and the heterogeneity of heparin leading to the early release of lower molecular weight chains.

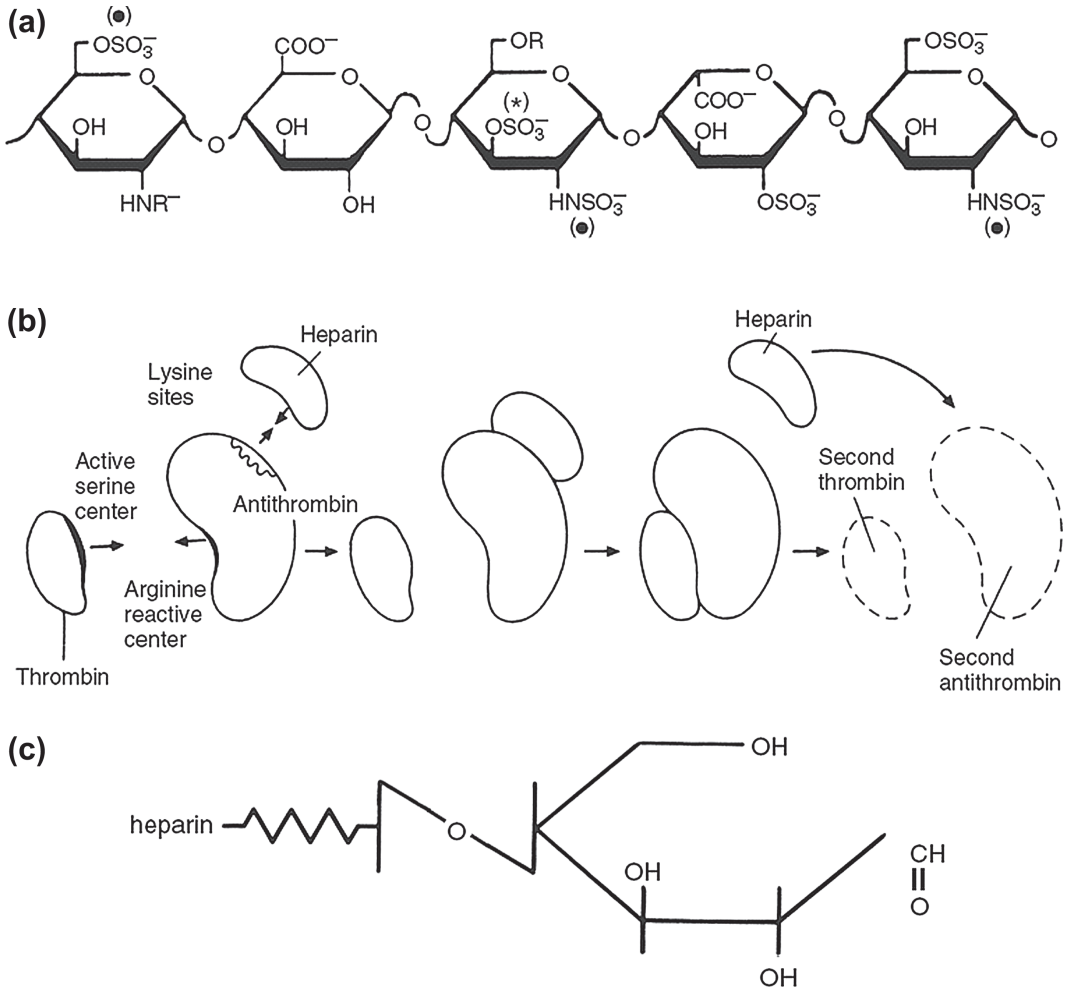


FIGURE II.5.2.10 (a) Antithrombin III-binding pentasaccharide of heparin. The pentasaccharide sequence is composed of three glucosamine (units 1, 3, and 5), one glucuronic acid (unit 2) and one iduronic acid (unit 4) units. Structural variants are indicated by –R′ (–H or $–SO_3–$) or –R″ ($–COCH_3$ or $–SO_3–$). The 2-O-sulfate groups (asterisk), a marker component for the antithrombin-binding region, and sulfate groups indicated by (•) are essential for high-affinity binding to antithrombin. (b) The mechanism of action of heparin and antithrombin III. (c) The reducing end of heparin formed by treatment with nitrous acid. *((a) from Fiore and Deykin, 1995; (b) from Bauer and Rosenberg, 1995, with permission.)*

The thromboresistance of heparinized materials based on controlled release appears to be due to a microenvironment of heparin in solution at the blood–material interface. The relationship between release rate (N, g/cm^{2}s) and surface concentration (C_s, g/cm^3), for heparin or any other agent released from the inside wall of a tube of radius r_0 is given by (Basmadjian and Sefton, 1983):

$$\frac{C_s}{Nr_0/D} = 1.22\left[\frac{x/r_0}{\mathrm{Re}Sc}\right]^{1/3} \tag{4}$$

where D = diffusivity (cm^2/s); r_0 = tube radius; x = axial position; Re = Reynold's number = $2r_0v\varrho/\mu$; Sc = Schmidt number = $\mu/\varrho D$; v = average velocity; ϱ = density; μ = viscosity. The diffusivity of heparin is ~7.5×10^{-7} cm^2/s and the critical C_s (minimum therapeutic level) for heparin is ~0.5 μg/mL.

Use of tridodecyl methylammonium chloride (TDMAC; Lehninger, 1966), a lipophilic, cationic surfactant, eliminated the need for the graphite coating that was part of the original graphite–benzalkonium–heparin (GBH) method (Gott et al., 1963). TDMAC and the many other quaternary ammonium compounds enabled heparin to be ionically bound to a wide range of biomaterials. Unfortunately, such compounds are surfactants with potentially toxic consequences, and are known to be eluted from the surfaces within a week.

Quaternizable amino groups have also been incorporated directly into polymers to improve the stablility of the ionically bound heparin. Tanzawa et al. (1973) synthesized a graft copolymer with a dimethylaminoethyl group by UV initiated copolymerization of N,N-dimethylaminoethyl methacrylate (DMAEM) and methoxypolyethylene glycol methacrylate (MPEG). Use of this technique (Angiocath™, Toray Industries) to coat poly(urethane) catheters indicated that a minimal heparin elution rate of 0.04 μg/cm^2/min was needed to render the catheter thrombus free *in vivo* (Idezuki et al., 1975). This value is consistent with Equation (4).

A commercial procedure (Baxter Bentley Healthcare Systems, Irvine, CA) to ionically bind heparin (Duraflo

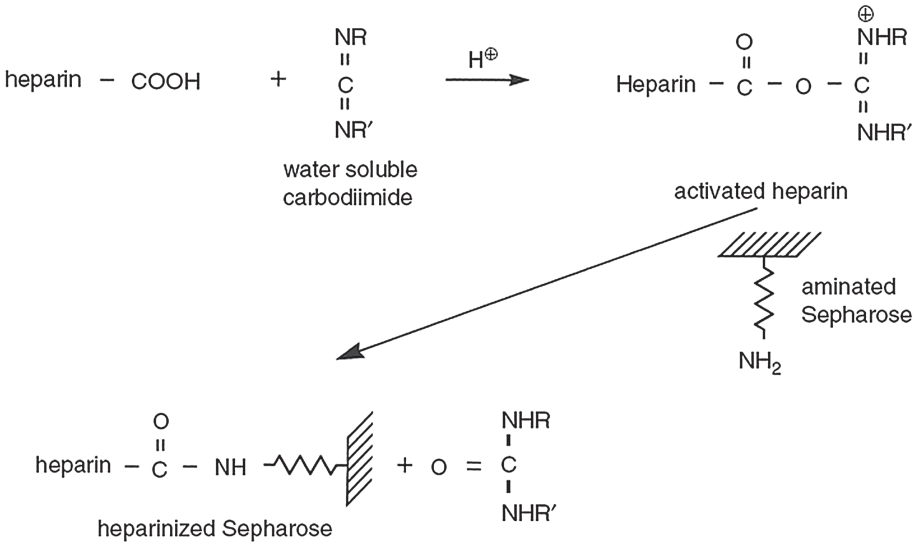


FIGURE II.5.2.11 One-step carbodiimide activation of carboxyl groups of heparin for binding to aminated Sepharose. For EDC, R = $(CH_2)3N(CH_3)_2$. For a carboxylated substrate (e.g., hydrolyzed polymethyl acrylate) its carboxyl groups may be activated by carbodiimide in a separate step for subsequent reaction with the free amine groups of heparin.

II™) has been used to coat cardiopulmonary bypass circuits and other devices. While heparin coating of extracorporeal circuits is designed to reduce surface thrombus formation, its effect on complement, contact activation, and inflammation is unclear and often contradictory (see below).

Covalently Bound Heparin. To impart a degree of activity longer than that possible with ionic linkages, heparin has been covalently immobilized to material surfaces. It is now recognized that the conformation of the attached heparin and the point of attachment (end point versus multipoint) are critical factors determining the catalytic efficiency of the immobilized heparin. Lindhout et al. (1995) studied the antithrombin activity of surface-bound heparin under flow conditions. They demonstrated that the rate of thrombin inactivation of the antithrombin–heparin surface equals the maximal rate of transport of thrombin toward the surface when the surface coverage of antithrombin exceeds 10 pmol/cm^2; thus indicating that a higher intrinsic catalytic efficiency of a surface does not necessarily result in a higher antithrombin activity.

Many of the coupling methods listed in Chapter I.2.17 have been used for heparin. For example, Heyman et al. (1985) attached 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC) activated heparin covalently onto chemically modified polyetherurethane catheters through a diamino alkane spacer. The immobilized heparin retained its ability to bind and inactivate thrombin and factor Xa. Unfortunately, carbodiimides (Figure II.5.2.11) are recognized as less than ideal activating agents for immobilizing heparin, since the acidic conditions used can result in a loss of anticoagulant activity.

Larm et al. (1983) developed a technique by which heparin can be covalently end point-attached (commercialized as the Carmeda® BioActive Surface, CBAS®) to the surface of plastics, as well as glass and steel. Heparin is first partially depolymerized by deaminative cleavage using nitrous acid to produce heparin fragments terminating in an aldehyde group (Figure II.5.2.10c). The heparin is then covalently linked to the primary amino groups of polyethylene imine (PEI) (Figure II.5.2.12a). This technique results in a highly stable, low thrombogenicity coating that has been demonstrated to retain its efficacy *in vivo* for four months in dogs (Arnander et al., 1987), and during patient treatment with an artificial lung (Bindslev et al., 1987). There was also a beneficial effect on *in vivo* bacterial colonization of treated polyurethane central venous catheters (Appelgren et al., 1996).

The commercial Trillium® coating (developed by Bio-Interactions Ltd and marketed by Medtronic) combines covalently binding heparin with polyethelene oxide and sulfonate groups (Figure II.5.2.12b). Clinical studies with Duraflo, Carmeda, and Trillium have shown that while they enable the use of reduced systemic anticoagulation (Ovrum et al., 2001), they do not reduce the post-operative acute phase response (van den Goor et al., 2004; Hoel et al., 2004). Complement and leukocyte activation play an important role in the biocompatibility of extracorporeal circulation devices, and it appears that the benefit of heparin coatings is limited to anticoagulation.

Heparin has also been covalently immobilized via its terminal serine at the end of the protein–carbohydrate linkage region to polyvinyl alcohol hydrogel. The heparin is bound during the Lewis acid catalyzed cross-linking of polyvinyl alcohol chains with glutaraldehyde. The heparinized polyvinyl alcohol hydrogel possessed significant anticoagulant activity, although the platelet incompatibility of the hydrogel substrate led to significant platelet consumption in an *ex vivo* canine shunt model (Cholakis et al., 1989).

Park et al. (1988) have covalently bound heparin to a polyurethane using PEG spacer groups. The increasingly mobile nature of the longer hydrophilic spacer chains were considered to have increased the observed bioactivity of

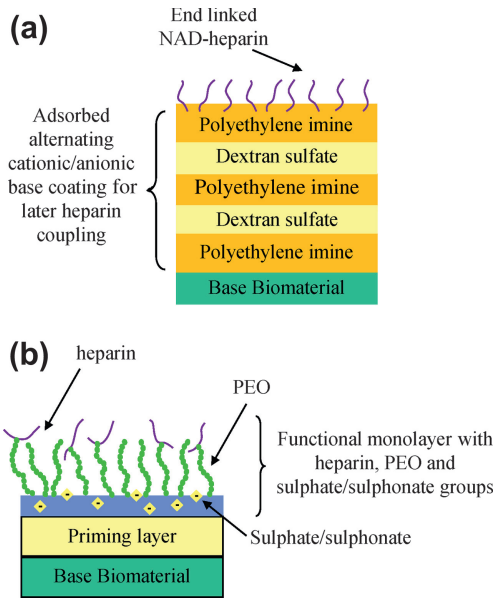


FIGURE II.5.2.12 Commercial surface treatments used for cardiopulmonary bypass (CPB) and potentially also for vascular grafts. (a) Carmeda method for immobilizing nitrous acid treated heparin (end-linked heparin) to dextran sulfate and polyethylene imine treated surface. (b) Trillium™ coating: The priming layer is hydrophilic and strongly binds to the base material, PEO chains and sulphate and sulphonate groups are covalently attached to the priming layer, while heparin is covalently bound to PEO. (*Adapted from Shivpal and Luthra, 2002.*)

immobilized heparin by providing a more bulk-like environment for the heparin. An intravascular oxygenator and carbon dioxide removal device (IVOX®; CardioPulmonics, Salt Lake City, UT) also uses covalent heparin bound to a PEG spacer. The PEG is grafted onto a plasma polymerized polysiloxane on a base material after plasma amination of the surface. Heparin has also been immobilized on silicone via an N-succinimidyl carbonate PEG and the grafted heterobifunctional PEG allowed for high heparin density resulting in both low fibrinogen adsorption and high specificity for ATIII (Chen et al., 2005).

Du et al. (2007) covalently grafted an antithrombin/heparin complex onto a PEO modified polyurethane in the presence of a free radical initiator. This increased anti-FXa activity compared to grafted heparin alone, and was shown to significantly reduce material thrombogenicity in a chronic rabbit model (Klement et al., 2006).

Thrombin Inhibition without Heparin

Recognizing the importance of inhibiting material-induced thrombin generation, agents other than heparin have been used to inhibit thrombin and thereby lower the thrombogenicity of the material. For example, hirudin, a potent thrombin inhibitor, has been covalently bound to biomaterials. Compared with heparin, hirudin is advantageous because it has no influence on platelet function, no immune-mediated platelet activating activity and most importantly, it does not require the presence of endogenous cofactors like antithrombin III. Thus, the polypeptide hirudin is thought to be able to block clot-bound thrombin that is inaccessible to inhibition by a heparin/antithrombin III complex. On the other hand, heparin functions as a catalyst. That is immobilized heparin, if not blocked by adsorbed protein, can continually accelerate the inactivation of thrombin by antithrombin III. It is unclear how long a covalently immobilized hirudin surface would remain effective, and even whether an immobilized hirudin could gain access to the clot-bound thrombin. Nevertheless, Ito et al. (1991) used heterobifunctional cross-linking reagents to derivatize hirudin to form covalent cross-links between hirudin and albumin, producing active conjugates for immobilization to surfaces. Others have immobilized hirudin to a polylactide–glycolide copolymer (Seifert et al., 1997). Another group has exploited controlled release of hirudin for this purpose (Kim et al., 1998). Small molecule thrombin inhibitors, like D-Phe-Pro-Arg chloromethyl ketone (PPACK) and others under development may prove useful as alternatives.

Because of the affinity of benzamidine toward thrombin and its potential to reduce complement activation (which may participate in platelet activation), amidine derivatives are currently being considered to enhance blood compatibility. Gouzy et al. (2004) immobilized a benzamidine derivative (with a hydrocarbon side chain) onto a maleic anhydide copolymer via a PEG spacer; platelet adhesion and activation was reduced on the modified surface, as well as thrombin–antithrombin complex formation. They subsequently correlated thrombin binding to the amount of immobilized benzamidine derivative, with the amount immobilized being dependent on spacer length and immobilization chemistry (Salchert et al., 2005). Yim and Sefton (2008) converted the surface nitrile groups of polyacrylonitrile-co-vinyl chloride films into amidine groups by first reacting nitrile with diethyl ether and hydrogen chloride followed by reaction with ammonia in ethanol (Pinner's synthesis). While the amidine surface modification was limited, reduced platelet adhesion and activation was observed, as well as a reduction (although not significant) in platelet microparticle formation.

Another approach involves immobilizing thrombomodulin, an endothelial cell associated protein that inhibits thrombin by activating protein C. Using a small scale dialyzer it was demonstrated that immobilized human thrombomodulin (on cellulose) still had co-enzyme activity for activation of protein C and anticoagulant activity (Kishida et al., 1995). An amino terminated silane was used to couple thrombomodulin to glass, and the authors reported both anticoagulant activity and reduced platelet adhesion (Han et al., 2001). Thrombomodulin has also been immobilized (using an azido group via PEG) onto pancreatic islets, with a view to minimizing the thrombogenicity of the islet and enhancing islet engraftment after portal vein infusion (Stabler et al., 2007). Further work is needed to appreciate the potential of such surfaces.

Immobilization of Anti-Platelet Agents

There has been considerable interest in incorporating anti-platelet agents into materials to lower material thrombogenicity, especially given the commonly observed adhesion of platelets on biomaterials. Endothelial cells lining the natural vessel wall help prevent thrombus formation by secreting anti-platelet agents such as nitric oxide (NO) and prostacyclin (PGI2). Thus, a biomaterial containing anti-platelet agents is consistent with our understanding of endothelial cell function. Prostacyclin is potent yet unstable, and efforts to maintain its biological activity during immobilization, coupled with concern for the ability of platelets in the bloodstream to be affected by the immobilized prostacyclin, has limited the enthusiasm for such a strategy. On the other hand, a simpler approach, which also more closely mimics the biological environment, is to incorporate PGI2 or NO into materials for release. Unfortunately, the inability to load sufficient drug into materials for release limits the materials' lifespan, and this approach may be limited to be used in short-term cardiovascular devices such as oxygenators, sensors, and catheters. A novel approach to overcome such limitations is to use endogenous NO donors to generate NO at the blood–material interface. NO is an anti-platelet agent and a potent inhibitor of smooth muscle proliferation. This approach is described in more detail below.

Despite these challenges, many investigators have incorporated anti-platelet agents into surfaces. For example, Ebert et al. (1982) immobilized prostaglandin F2α using a diaminododecane spacer arm. The F2α was subsequently converted to the unstable prostacyclin. The benefits of spacer arms are described elsewhere (Chapter I.2.17). A related approach is to add an anti-platelet agent, such as prostaglandin E1 (PGE1), in addition to heparin into a polymer. A PGE1-heparin compound was synthesized and incorporated into a polyurethane (Jacobs et al., 1985). Also, prostacylin has been incorporated into polymer matrices for controlled release (McRea and Kim, 1978). Aspirin, capable of inhibiting the generation of the platelet activator thromboxane A2, has also been incorporated into polyvinyl alcohol membranes used in hemodialysis (Paul and Sharma, 1997), while a dipyrimadole (Persantin) derivative was photo-immobilized on a polyurethane (Aldenhoff et al., 1997).

Two basic approaches exist to create NO releasing polymers: N-diazeniumdiolates or S-nitrosothiols (Figure II.5.2.8c,d). These can either be blended into polymers or covalently bonded to a side chain or to the polymer backbone (Frost et al., 2005). While S-nitrosothiol-based NO releasing materials have been synthesized (e.g., with polyvinyl alcohol (PVA) and PVP (Seabra and de Oliveira, 2004)), there is currently only limited biocompatibility data on such an approach (Bohl and West, 2000). On the other hand, NO-releasing materials using diazeniumiolates have demonstrated great efficiency in reducing thrombus formation in various animal models (Frost et al., 2005; Skrzypchak et al., 2007). Another approach is to use endogenous NO donors to produce NO locally. Duan and Lewis (2002) used endogenous S-nitrosoproteins in plasma to produce NO from immobilized cysteine to minimize platelet adhesion on a polyurethane and a poly(ethylene terephthalate). A lipophilic copper complex has also been incorporated into polyvinyl chloride (PVC) and polyurethane (PU) films that was able to generate NO from nitrite under physiological conditions (Oh and Meyerhoff, 2004).

A new generation of anti-platelet agents based on inhibiting fibrinogen binding to activated platelet GPIIb/IIIa receptors offers a fresh approach to lowering the thrombogenicity of surfaces via drug release. These agents, from blocking monoclonal antibodies to small peptides and compounds (e.g., DMP728, Figure II.5.2.9), not only block platelet aggregation, but will also likely block platelet adhesion to artificial surfaces. Some have already been incorporated into stents for release (see Section "Strategies to Lower the Thrombogenicity of Metals," below, and Chapter II.5.3.B).

Immobilization of Fibrinolytic Agents

Some investigators have sought to promote fibrinolysis on artificial surfaces by the surface generation of plasmin. Clot lysis is achieved by the action of plasmin (formed from plasminogen) on fibrin. However, the action of such surfaces may be "too little too late." Sugitachi and Takagi (1978) immobilized urokinase, a fibrinolytic enzyme that acts on plasminogen, on various materials. A slightly different approach is to immobilize plasminogen and then convert it to plasmin, so as to impart fibrinolytic activity to a surface (Marconi et al., 1996). A disadvantage of utilizing streptokinase and urokinase to cleave plasminogen to plasmin is that these agents activate both circulating and fibrin-bound plasminogen. This contrasts with the action of tissue plasminogen activator (tPA), an endogenous serine protease that converts only fibrin-bound plasminogen to plasmin. A nonpharmacological approach to developing fibrinolytic surfaces for blood-contacting applications was the preparation of lysine-derivatized polyurethane surfaces (Woodhouse and Brash, 1992). The expectation was that these surfaces exhibit fibrinolytic activity because the lysine residues promote the selective adsorption of plasminogen from plasma through the lysine-binding sites in the plasminogen molecule.

USE OF ENDOTHELIAL CELLS AND RGD PEPTIDES

It is intuitive to believe that the ideal nonthrombogenic surface for vascular grafts and other devices will consist of an intact luminal endothelial cell layer. Herring et al. (1984) seeded Dacron™ and ePTFE grafts with endothelial cells in a preliminary clotting step with blood and endothelial cells. Autologous endothelial cells were seeded onto ePTFE grafts (6–7 mm inner diameter) for 153 patients, and a significant improvement in the

patency rate in the infrainguinal position at seven years was reported (Meinhart et al., 2001).

To promote endothelialization of vascular grafts either *in situ* or *in vitro*, the role of surface chemistry, pore size, and protein coatings have been investigated (McGuigan and Sefton, 2007). Using a rat model, Wang et al. (2004) found increased endothelialization in porous polyurethane grafts. To increase endothelial cell attachment and proliferation, surface modification using an anhydrous ammonia gaseous plasma technique (Sipehia, 1990; Pu et al., 2002) or UV irradiation (Olbrich et al., 2007) has proven successful *in vitro*. Some approaches are focused on modifying the surface with proteins or peptides that will promote adhesion and proliferation of endothelial cells. Coating or covalent binding of materials with extracellular matrix (ECM) proteins such as fibronectin (Van Wachem and Hubbell, 1987), collagen, laminin, and elastin have shown some success *in vitro*. A more elegant approach (Massia and Hubbell, 1990) has been to immobilize RGD ECM peptide sequences, (see Chapter I.2.17) to encourage endothelial cell attachment. This approach has been adopted by many others, but most results remained at the proof-of-concept stage with *in vitro* studies (for a thorough review of the potential use of peptides to enhance vascular graft endothelialization, refer to de Mel et al., 2008).

Immobilizing growth factors such as VEGF, ECGF, or a metalloproteinase inhibitor have also been shown to promote endothelial cell proliferation on graft materials *in vitro*. It is important to note that while graft endothelialization might occur from cell migration from the anastomosis or from infiltration, it is thought that endothelial progenitor cells (EPC)[3] may also play a role in graft endothelialization, and thus increasing the mobilization of EPC through surface modification (e.g., CD34 immobilization; Aoki et al., 2005) is another approach.

Since considerable differences exist in the potential for endothelialization between humans (low) and animals (higher, e.g., canines), translation of results from animal studies can be problematic. A detailed discussion of this issue is presented by Zilla et al. (2007). With immobilizing adhesion peptide sequences, there is a concern that regions not covered by endothelial cells would lead to enhanced platelet and thrombus deposition, or that the attached endothelial cells might indeed be activated (see Box 3).

STRATEGIES TO LOWER THE THROMBOGENICITY OF METALS

While polymers represent the principal class of materials used in cardiovascular setting, metallic surfaces are also used in mechanical heart valves and stents. Most metals are highly thrombogenic and life-long anticoagulant and anti-platelet therapies are required with the use of mechanical heart valves while the optimum therapy for stented patients is a much discussed topic, especially with drug eluting stents (see Box 4).

To reduce the thrombogenicity of the materials used in prosthetic heart valves, three approaches for surface modification have been explored: coating whereby a layer of metal oxide is deposited on the metal (plasma deposition); oxidation of the metal; and ceramic or polymer coating. Deposition of tantalum-doped titanium oxide films significantly reduced platelet activation and fibrin deposition in an *in vivo* dog model (Huang et al., 2003). Coating with oxynitrites on stainless steel also improved overall *in vitro* and *in vivo* biocompatibility (Chen et al., 2003). A layer of oxide on titanium has also been prepared by oxidation in a solution of H_2O_2, and this reduced platelet adhesion *in vitro* (Takemoto et al., 2004). Diamond-like carbon (DLC) (Mikhalovska et al., 2004) and apatite composite (Muramatsu et al., 2003) have also been coated on various metals, and both a reduction in fibrinogen adsorption and platelet adhesion have been observed *in vitro*. More recent approaches focus on coatings with multilayers of collagen and sulfated chitosan (Li et al., 2008), or covalently

BOX 3 Associated Facts: Endothelial Cell Activation on Vascular Grafts

When endothelial cells (EC) are expected to adhere onto vascular grafts (or any cardiovascular material) to provide a nonthrombogenic surface, it is required that the cells express a non-activated, nonthrombogenic phenotype, such that leukocytes and platelets do not bind to them and the endothelial cells are able to inhibit coagulation and platelet activation. While many investigators measure endothelial cell adhesion to a substrate, few characterize the actual phenotype of the adherent cells (McGuigan and Sefton, 2007). It is not a simple matter to determine adherent EC thrombogenicity *in vitro*, since cell seeding density, the cell origin (e.g., adipose tissue versus umbilical vein), shear rate, and culture conditions (serum versus no serum, human versus bovine serum) seem to affect the expression of ICAM-1, VCAM-1 (leukocyte adhesion receptors), PECAM-1 and E-selectin (platelet adhesion receptors), and the effects are material dependent. Difference in the *in vivo* endothelialization process between humans and animals (e.g., canines) further complicates matters (Zilla et al., 2007).

BOX 4 Area of Further Interest: A Benefit of "Poor" Material Blood Compatibility

While metallic stents are being modified to reduce their potential thrombogenicity, thrombogenicity may be beneficial to the promotion of osteogenesis with orthopedic implants. Upon implantation, osteogenic cells interact first with the blood clot that occupies the wound site, and platelet activation releases growth factors, such as PDGF, and cytokines, such as TGFβ (both contained in platelet's α granules), that are recognized to stimulate bone growth. As such, platelet concentrates are currently being used during surgery to improve bone regeneration. More generally, thrombogenicity is a special case of inflammation (Gorbet and Sefton, 2005b), and enhancing thrombogenicity may be a strategy for creating materials with improved healing characteristics in situations such as in orthopedics.

[3]EPC are circulating bone-derived progenitor cells. They circulate in blood at low concentration and share some similarities with monocytes.

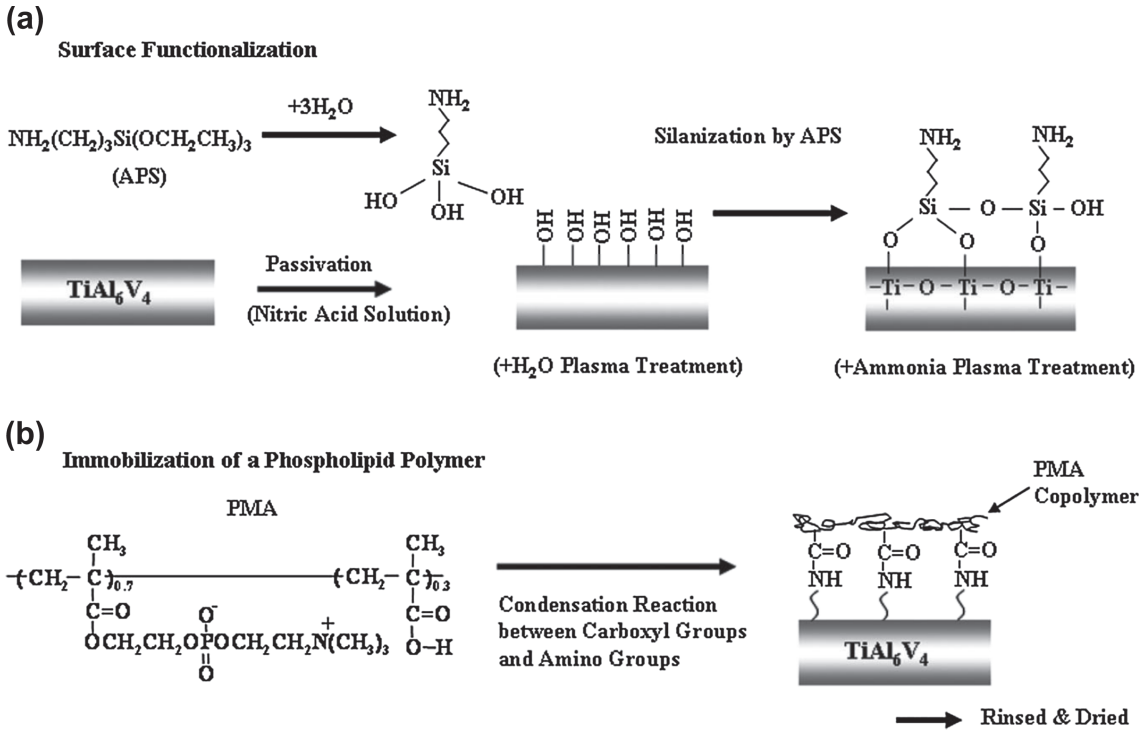


FIGURE II.5.2.13 Immobilization of a phospholipid polymer (PMA: poly(2–methalcryloyloxyethylphosphorylcholine-co-methacrylic acid)) on Ti-6Al-4V. *(From Ye et al., 2008.)*

attaching a phosphorylcholine containing polymer (see Figure II.5.2.13; Ye et al., 2008). Despite these efforts, low temperature isotropic pyrolytic carbon remains the choice for heart valves (see Chapter II.5.3.A).

Developing stent coatings for the metals currently used (stainless steel, cobalt chromium, tantalum, and Nitinol) in bare metal stents were sought as a means of reducing the thrombosis rates of such stents (Van Belle et al., 2007), and to at least indirectly influence restenosis. Due to its inertness, gold was deposited on stents, but increased re-stenosis rates were observed (Hara et al., 2006). Randomized clinical trials of heparin-coated tantalum stents (Blezer et al., 1998) showed that there was no significant improvement in re-stenosis or clinical outcomes compared to uncoated stents (Wohrle et al., 2001). Other efforts to reduce thrombogenicity included coating tantalum stents with polymers such as polyetherurethane and parylene to reduce platelet adhesion density (Fontaine et al., 1996) and radiation grafting N-vinyl-pyrrolidone (NVP) onto plasma-treated stainless steel stents (Seeger et al., 1995). Hyaluronic acid coating also reduced platelet thrombus formation on stainless steel stents and tubes in a primate thrombosis model (Verheye et al., 2000). In another study, metal/polymer composite stents loaded (40% by weight) with a potent anti-platelet agent (GPIIb/IIIa antagonist; >90% elution in 89 hours) reduced, by almost a factor of 2, platelet adhesion in dogs two hours after stent deployment (Santos et al., 1998). r-hirudin has also been immobilized on Nitinol stents coated with functionalized paracyclophanes (Lahann et al., 2001). As with vascular grafts, a phosphorylcholine coating has also been explored to improve stent endothelialization with BiodivYsio™ (Biocompatibles, Surrey, UK), a PC-coated stainless coated stent. However, it did not show significant improvement compared to bare metal stents in clinical trials (Abizaid et al., 2007). The relationship between thrombogenicity and clinical outcomes such as re-stenosis remains a hypothesis.

The early work on reducing stent thrombosis has been supplanted by drug-eluting stents (DES) to reduce re-stenosis rates. Rapamycin or sirolimus eluting stents in which the therapeutic agent is blended in a nonerodible polymer and coated onto the surface of the stent have the dramatic effect of eliminating in-stent re-stenosis for at least six months after deployment (Morice et al., 2002). Paclitaxel is an antineoplastic agent used in chemotherapies. However, the concern that DES appear to have higher risk of late thrombosis (Box 5) has renewed interest in developing surface strategies to lower stent thrombogenicity. Also, because of late thrombosis, there has been an interest in biodegradable stent materials. A drug delivery stent based on phosphorylcholine coating releasing zotarolamus (Endeavor ZES, Medtronic) performed well in clinical trials and received US Food and Drug Administration (FDA) approval recently (Slottow et al., 2007). Full and early endothelial coverage of the stent would also potentially address both the issues of re-stenosis and thrombosis. For example, a covalently coupled polysaccharide coating with an antibody (anti-CD34) to bind circulating endothelial progenitor cells has been deposited on stainless steel stents with positive initial clinical reports (Aoki et al., 2005). Other means of promoting endothelialization (see Section "Use of Endothelial Cells and RGD Peptides") are also under active investigation for stents.

BOX 5 Clinical Correlations: Drug Eluting Stent Induced Thrombosis

Drug eluting stents (DES) have significantly reduced the rates of re-stenosis. However, clinical data, albeit not without dispute, suggests that DES increase the risk of late thrombosis (>30 days after implantation). While changes to the antithrombotic and anticoagulant regimen have resolved some of the concerns, ongoing animal and *in vitro* research (Lüscher et al., 2007) has suggested that in addition to preventing neointima formation (the intended use of the therapeutic agent being delivered), paclitaxel and sirolimus inhibit re-endothelialization and induce tissue factor, thus creating a prothrombotic environment at the local site of stent implantation. The proprietary nondegradable polymers used in these stents have also been reported to be linked to inflammatory reactions at the site of implantation, although it is not clear whether these are different from what is seen with metals. A chronic inflammatory reaction would further contribute to a prothrombotic state around the stent. The clinical concerns have driven a resurgence in stent endothelialization strategies.

SUMMARY

It is an axiom that the interactions between materials and blood are complex. Hence, it is no surprise that developing low thrombogenicity materials (let alone ones with zero thrombogenicity) are challenging. Medical device manufacturers relying on elegant device designs and systemic pharmacological agents have significantly improved existing materials. Adverse effects are minimized and existing devices, if not risk-free, provide sufficient benefit to outweigh the risks. The focus of research in biomaterials is to make better materials that have fewer risks and greater benefits. Stents that "actively" prevent re-stenosis are a great example of how modifying a material can have a dramatic clinical effect. Inert materials, such as those with immobilized PEG, can resist protein and platelet deposition, but these may only be at best surrogate markers for thromboembolic phenomena. On the other hand, incorporating anticoagulants such as heparin can be an effective means of reducing thrombin production rates below critical values (e.g., $k_p < 10^{-4}$ cm/s), but this may not be sufficient to prevent platelet activation and consumption. Many strategies for lowering thrombogenicity have been identified, and they all show a beneficial effect in at least one assay of thrombogenicity. However, few if any have made the transition from a one-parameter benefit to multiple benefits, or from *in vitro* to *in vivo*. These issues are discussed more fully in Chapters II.2.6 and II.3.5.

Which approach will ultimately be successful is impossible to predict. Certainly there is much activity in biomembrane mimicry and PEG immobilization. There are many new anticoagulants and antithrombotics under development, but few have yet to be incorporated into material surfaces. In recent years, combining strategies such as reducing both protein adsorption and thrombin production have become prevalent. The commercial Trillium® coating is an example of the benefit of combining various approaches. Finally, as new hypotheses are developed to understand cardiovascular material failure, new approaches will be identified for inhibiting undesired pathways. The failure to produce the ideal nonthrombogenic material, despite over 30 years of research, merely reflects our limited understanding of blood–material interactions (Zilla et al., 2007; Ratner, 2007). Perhaps the right strategy for producing a nonthrombogenic material will have little to do with controlling platelets or thrombin, but will be directed towards leukocytes, endothelial cells, or complement (Gemmell, 1998; Wetterö et al., 2003). Ongoing research is expected to improve the blood interactions of materials used in medicine to address important clinical needs.

CASE STUDY Nonthrombogenic Materials and Strategies

Michael V. Sefton and Maud B. Gorbet

ISO10993-4, Biological Evaluation of Medical Devices Part 4: The Effects on Blood (AAMI, 1995), which manufacturers of medical devices need to use as guidance to register their products, includes thrombosis and coagulation among the tests that need to be done. However, specific test methods are not detailed. With a view to clarifying this question, a series of plasma-modified tubes (along with an unmodified control and other commercially available tubing) were prepared, surface characterized, and exposed to heparinized whole blood (1 U/mL heparin) for one hour at 37°C (Sefton et al., 2001). The surface modifications included several different plasma vapors (H_2O, CF_4, and fluorine). The 1.5 mm ID tubing was incubated with whole blood on a rocking platform to gently agitate the blood and keep the cells from overtly settling. This system does not probe the effect of shear on cell activation; rather the agitation and long incubation time are thought sufficient to create "well-mixed" conditions.

Some of the results from this study are shown in Figure II.5.2.14 and Table II.5.2.1. One of the conclusions from this study is that the materials with the lowest levels of platelet and leukocyte activation (microparticle formation, CD11b upregulation) were the unmodified materials (polyethylene, Pellethane™, a polyurethane), and that the surface modifications tested here had either no effect or only made things worse from the perspective of platelet and leukocyte activation. The scanning electron micrographs showing little cellular deposit on the polyethylene or Pellethane™ were consistent with the flow cytometry findings. Except for those materials that were worse, the other materials expressed similar levels of activation for platelets, leukocytes, and the other markers tested; i.e., the majority of the "inert" materials (in the absence of bioactive components like heparin) appear to have a similar non-specific effect on blood.

The results from this study contrast sharply with other studies (many of them cited in this chapter) showing large differences in blood interactions between different biomaterials. This highlights the lack of consensus about blood–materials interactions. Reasons for this lack of consensus are discussed in Chapter II.3.5.

Continued

CASE STUDY **Nonthrombogenic Materials and Strategies (continued)**

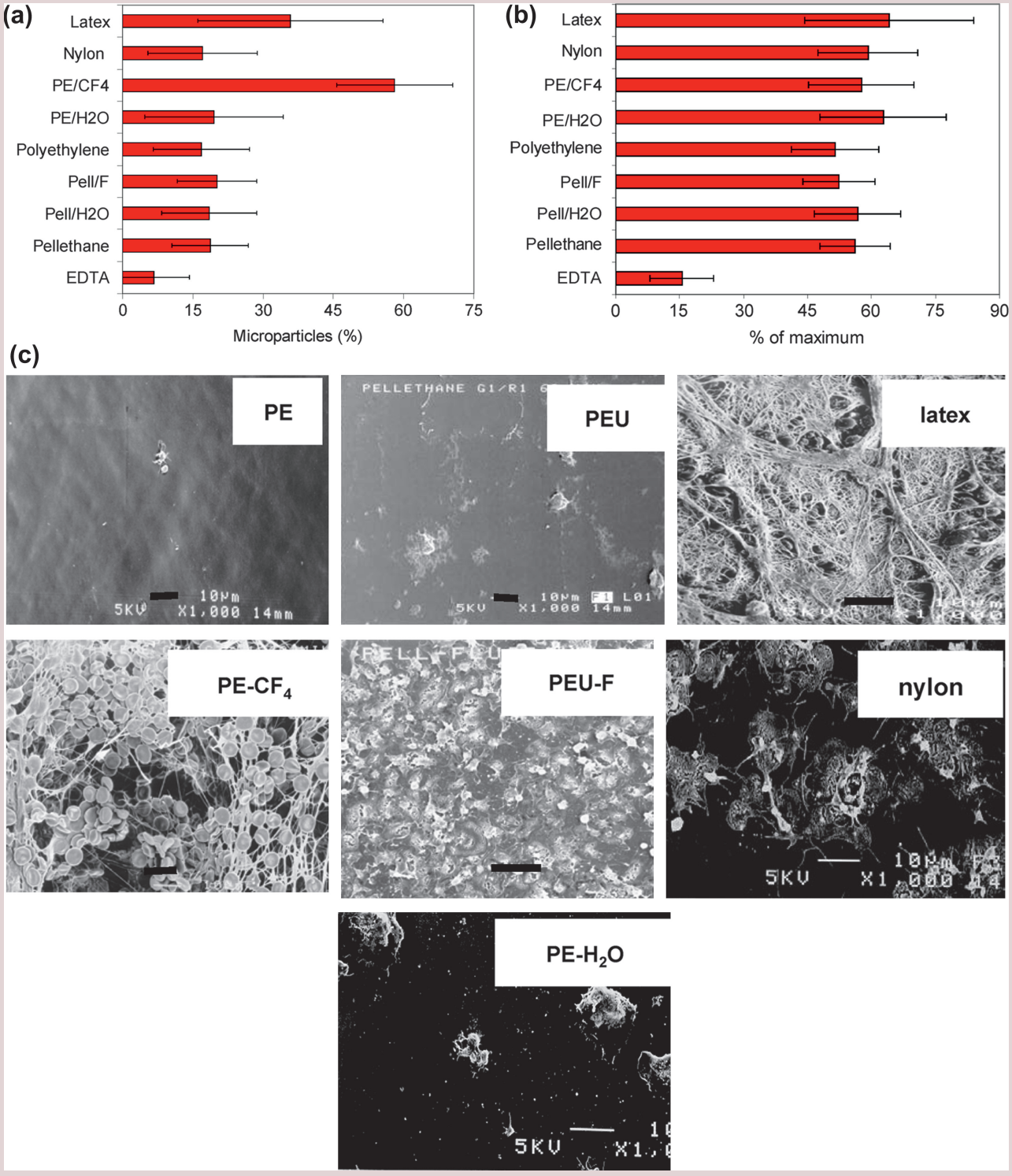


FIGURE II.5.2.14 Cell activation and SEM (scanning electron microscope) results after 60 min contact with whole blood (1 U/mL heparin) at 37°C in rocking platform. Flow cytometry results are mean ±SD. (a) Microparticle levels (percentage of platelet events); (b) Leukocyte CD11b upregulation (expressed relative to the maximum upregulation obtained with a phorbol ester). (c) SEM. Scale bar is 10 μm in all. Modified materials are designated with name of base materials and gas introduced into plasma (H_2O, NH_4, CF_4, or F). Presented data is a subset of those studied and figures are adapted from those published (Sefton et al., 2001). PE: polyethylene, PEU: Pellethane.

Continued

CASE STUDY **Nonthrombogenic Materials and Strategies (continued)**

TABLE II.5.2.1 Most Biomaterials are the Same

Parameter	EDTA Control (or Equivalent)	Value for Most Materials
Platelet count loss (%)	0 (by definition)	25–35
P-selectin (% positive)	6.4	~8–9
Platelet–leukocyte aggregates (fluorescent intensity)	46	~200–250
CD11b upregulation (% of maximum)	15	~50–60
L-selectin shedding	11	~70–90

Most materials, despite very different non-specific chemical modifications, resulted in similar levels of platelet and leukocyte activation. These were higher than the corresponding negative control values. The similarity of CD11b upregulation values is seen in Figure II.5.2.1b, while the presence of exceptions that were more activating is seen in the microparticle results in Figure II.5.2.1a.

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